Hetero-oligomeric tagging diminishes non-specific aggregation of target proteins fused with Anthozoa fluorescent proteins

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The tendency for tetramerization is the main disadvantage in the green fluorescent protein homologues from Anthozoa species. We report a universal method called hetero-oligomeric tagging, which diminishes troublesome consequences of tetramerization of Anthozoa-derived fluorescent proteins (FP) in intracellular protein labelling. This approach is based on the co-expression of the FP-tagged protein of interest together with an excess of free non-fluorescent FP mutant. The resulting FP heterotetramers contain only a single target polypeptide and, therefore, can be considered pseudo-monomeric. Feasibility of the method has been demonstrated with a red FP fused with cytoplasmic β-actin or tubulin-binding protein Tau34. In addition, heterotetramers appeared to be a unique model for biophysical characterization of Anthozoa FPs in pseudo-monomeric state.

Key words: coral fluorescent protein, green fluorescent protein, heterotetramer, protein labelling, red fluorescent protein, tetramerization.

INTRODUCTION

A green fluorescent protein (GFP) from jellyfish *Aequorea victoria* has become a popular tool for many biological and biotechnological purposes and countless mutant variants with altered properties have been generated for research purposes [1]. The colour pallet of GFP-like proteins has been considerably expanded by Anthozoa-derived fluorescent proteins (FPs) [2]. An intrinsically bright red FP, termed DsRed, with a fluorescent maximum at 583 nm, soon became commercially available. Also, several other red-emitting FPs were generated [3–5]. In particular, a bright mutant M355NA with emission at 595 nm was obtained on the base of a naturally non-fluorescent asFP595 (asulCP) chromoprotein [6].

However, it has now become obvious that a number of features narrow the application field of Anthozoa FPs, the most vivid problem being strong tetramerization tendency. Multiple experiments provide a distinct line of evidence of DsRed existing as a non-dissociable tetramer both *in vitro* and *in vivo* [7–10]. Similar characteristics were obtained for other species [5,6,10].

Recently [11], a method was described that allows the rescue of an otherwise non-functional protein complex, assembled with DsRed-tagged subunits. The method relies on expressing excessive amounts of target protein within the same cell as DsRed-tagged target. Free target molecules compete with DsRed-fused targets in the process of specific target oligomerization. As a result, protein aggregation that occurs owing to tag interaction is being impeded. Inspired by this idea, we developed a novel approach to convert tetrameric FPs into psedo-monomeric state.

EXPERIMENTAL

Plasmid construction

To construct M355NA-actin fusion, the full-length coding region of M355NA without stop codon was cloned into a pEGFP-actin vector (ClonTech, Palo Alto, CA, U.S.A.) between *Age*I and *Bgl*II restriction sites, in place of the EGFP-coding region. The Tau34 coding region was amplified with primers 5′-TTTCTCGAGGGATGGCTGAGCCCCGC and 5′-GGGAGATCTTCACAAACCCTGCTTGG (restriction sites are underlined) using Marathon-Ready Human Brain cDNA (ClonTech) as a template, and cloned into M355NA-actin vector between *Xho*I and *Bam*HI restriction sites, in place of the actin-coding region. Fusion proteins contained the four amino acid linkers, RTRA, between M355NA and the target polypeptide.

Site-directed mutagenesis was performed by overlap-extension method [12].

Internal ribosome-binding site (IRES) sequence was derived from pIRES2-EGFP vector (ClonTech). IRES-M355NA fusion sequence was created by the overlap-extension method. IRES-containing fragment was PCR-amplified from pIRES2-EGFP with a plasmid primer 5′-AGGCGTGTACGGTGGGAG and 5′-*GGAGGCCATGGTTGTGGCC*ATATTATC, which corresponds to both IRES and M35-5 sequences. The amplified part of multiple cloning sites was used for subsequent cloning. M355NA-containing fragment was PCR-amplified with primers 5′-*GGCCACAACCATGGCCTCC*CTGCT and 5′-CTCAAGATCTGTTGTGGCCCAGCTTGG (*Bgl*II restriction site is underlined). The overlapping regions of the two long primers is in italics. The resulting fragment was cloned into pEGFP-actin using *Nhe*I and *Bam*HI cloning sites. To create the complete vector, asulCP-NF1 sequence cloned in pEGFP-C1 in place of the EGFP-coding region was cloned into the construct described previously, using *Nhe*I and *Bgl*II restriction sites.

Cell culture

L929 and Chinese-hamster ovary (CHO)-K1 cells were obtained from A.T.C.C. and cultured in Dulbecco's modified minimal

Abbreviations used: CHO, Chinese-hamster ovary; FP, fluorescent protein; GFP, green FP; HO, hetero-oligomers; IRES, internal ribosome-binding site.

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essential medium (DMEM; Invitrogen, Carlsbad, CA, U.S.A.) and 50/50 % DMEM/F-12 media mixture (Invitrogen) respectively, supplemented with 10 % foetal bovine serum (Sigma). Cells grown to 50–70 % confluence on 18 mm × 18 mm coverslips in 35 mm dishes (Falcon, Lincoln Park, NJ, U.S.A.) were transfected with the above plasmids, using LipofectAMINE PLUS (Invitrogen) or FuGENE 6 (Roche, Indianapolis, IN, U.S.A.) reagents for L929 or CHO-K1 cells respectively. Cells were washed, 48 h after transfection, with Dulbecco's PBS and fixed with 4 % (w/v) paraformaldehyde in PBS for 30 min. For fluorescence microscopy, coverslips were mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.).

Fluorescence microscopy and image analyses

Images of fixed cells were acquired with an ORCA-ER digital camera (Hamamatsu) attached to an Eclipse E800 microscope (Nikon) equipped with Plan Apo 100 × /1.40NA oil immersion objective. Standard GFP–FITC (excitation, 460–505 nm; emission, 510–560 nm) and G-2A (excitation, 510–560 nm; emission, long-pass 590 nm) filter sets were used for EGFP and M355NA respectively. Exposure times of 0.48–4.56 s and high-resolution 1 × 1 binning mode of the camera were employed. AquaCosmos software (Hamamatsu) was used for processing digital images.

Protein expression and purification

For prokaryotic expression of M355NA and asulCP-NF2, full-length coding regions were cloned into the pQE30 vector (Qiagen, Chatsworth, CA, U.S.A.). Proteins fused to an N-terminal His_6 tag were expressed in *Escherichia coli* XL1 Blue strain (Invitrogen) and purified using the TALON metal-affinity resin (ClonTech). To purify hetero-oligomers (HOs) and M355NA from mammalian cells, 15 dishes of CHO-K1 for each construct were transfected by a vector encoding M355NA with His_6 tag at the N-terminus alone or co-transfected with a 50-fold excess of asulCP-NF1 or asulCP-NF2. After 48 h, the cells were washed with Dulbecco's PBS and detached with 0.05 % trypsin and 0.53 mM EDTA mixture (Invitrogen). After centrifugation the cells were lysed by sonication, and HOs and M355NA were further purified as described above.

SDS/PAGE and Western blotting

Proteins purified from mammalian cells were concentrated 10-fold using Ultrafugal-0.5 filter device (Millipore, Bedford, MA, U.S.A.). Non-heated samples were loaded on to a native SDS/10 % polyacrylamide gel, and electrophoresis was performed at 15 mA/gel. Gels were stained with Coomassie Blue. For Western blotting, proteins were transferred on to Hybond C membrane (Amersham) using standard procedures. Membranes were probed with mouse antibodies (ClonTech) against His_6 tag (1:1000), and then with horseradish peroxidase-conjugated anti-mouse antibodies (Amersham). A TMB-kit (Vector) was used to develop the staining pattern.

Spectroscopy

Absorption spectra were recorded with Beckman DU520 UV/VIS spectrophotometer. To determine molar absorption coefficients, M355NA and asCP-NF2 absorption spectra were measured for protein samples of more than 95 % purity (determined from SDS/PAGE). Then, maximal absorption intensities at 568 nm were normalized on protein concentrations measured by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.). A Varian Cary Eclipse fluorescence spectrophotometer was used for measuring excitation–emission spectra. Steady-state anisotropy was measured using a fluorescence spectrophotometer F-2500 (Hitachi) equipped with two polarizers in a 0.25 ml quartz cuvette. The samples contained 50 % glycerol in 100 mM Tris/HCl buffer (pH 8.0). Anisotropy calculations were performed as described previously [13]. All measurements were at room temperature (21 °C).

RESULTS

Outline of the method

The method is based on simultaneous *in vivo* expression of any particular Anthozoa FP in two forms. The first form is an FP in fusion with the protein of interest. The second form is free FP (helper FP). We proceeded from a consideration that free monomers and target-bearing monomers can assemble together and produce heterotetramers (Figure 1). Interactions between target-fused and free monomers should effect tetramers with 1, 2, 3 or 4 FP-fused target protein molecules reducing their quantity to one per tetramer at best. This becomes possible provided a considerable excess of free FP molecules is present within the cell. Thus a pseudo-monomeric FP tag is created, which prevents excessive cross-linking and aggregate formation.

The pool of extra FP molecules should create a considerable fluorescence background, interfering with target protein visualization. To resolve this problem, an 'invisible', non-fluorescent FP mutant should be used. Alternatively, a free helper can possess a different fluorescence colour as opposed to the fused FP. For instance, green-free GFP mutant can be used together with red FP-target fusion.

To co-express the free FP and target-fused FP in a eukaryotic cell line, simultaneous transfection with two vectors can be used. The vector encoding free FP should be used in excess. Alternatively, one can utilize an IRES-containing vector, which allows for bicistronic protein expression in eukaryotic cells. As translation of both proteins occurs from a single RNA molecule, one is protected against uneven cell-to-cell protein distribution. Cap-dependent translation is several times stronger when compared with IRES-dependent translation [14], so the FP-target fusion should be subjected to IRES-dependent translation to ensure excess free FP.

Generation of non-fluorescent mutants

We applied our method to a red fluorescent mutant protein M355NA. At first glance, the best free non-fluorescent partner for M355NA is the wild-type chromoprotein asulCP. However, asulCP is capable of a unique photoconversion: this protein becomes red-fluorescent under intensive green light irradiation. Thus excess asulCP should create a considerable background. To resolve this problem, we generated two completely non-fluorescent mutants: asulCP-NF1 and asulCP-NF2. Mutant asulCP-NF1 contained a substitution $Tyr^{64} \rightarrow Cys$ (Y64C) (Y66C in accordance with GFP numbering) that destroyed the chromophore. Obviously, this substitution might be applied to any other FP to transfrom it into a non-FP. The key substitution in the mutant asulCP-NF2 was A143C (A148C in GFP numbering) that led to a loss of the ability to be kindled (detailed report on the influence of some mutations on asulCP kindling properties will be published elsewhere). Thus asulCP-NF2 is a chromoprotein with no detectable fluorescence. Additionally, both mutants contained substitutions K6T and K7E to avoid

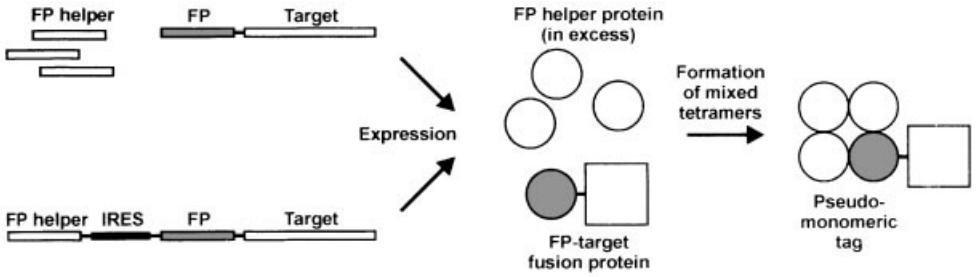

Figure 1 Schematic representation of the HO tagging method

Simultaneous expression of an FP-target fuse together with the excess of free non-fluorescent FP mutant (helper) results in the appearance of mixed tetramers that mimic a monomeric tag.

non-specific protein aggregation (these mutations were shown to resolve the problem of aggregation for M355NA [6]).

β-Actin labelling

To demonstrate feasibility of the method, human cytoplasmic *β*-actin was chosen as a model target protein. Functional EGFP-actin fusion protein was used to visualize the desired fluorescent pattern. Simultaneous transient co-transfection of cells with plasmids expressing both EGFP-actin and M355NA-actin constructs allowed the comparison of green and red fluorescent images within the same cell and, thus, the estimation of any imperfections in the red tag used. To determine the optimal excess of free helper, asulCP-NF1 or asulCP-NF2 expressing plasmid was added to a cell transfection in varied amounts. Three ratios of helper/M355NA-actin plasmids were tested: 3:1, 10:1 and 50:1.

The initial M355NA-actin fusion produced high levels of cytoplasmic aggregation within L929 fibroblasts (Figure 2A). Practically, all red fluorescent signals were concentrated into massive aggregates. These aggregates often produced both red and green fluorescence, probably due to co-aggregation of M355NA- and EGFP-tagged actin (EGFP-actin alone never forms aggregates). Addition of the excessive asulCP-NF1 resulted in a significant improvement of red fluorescent images proportional to the amount of helper protein added (Figures 2B–2D). Threefold helper excess led to a significantly reduced aggregation tendency of M355NA-actin and its more even cytoplasmic distribution. At the same time, incorporation of M355NA-actin into fine actin structures was very poor (Figure 2B). Tenfold excess of asulCP-NF1 ensured good distribution of red-labelled actin although aggregates were still present (Figure 2C). Finally, when the 1:50 ratio was examined, the pattern of actin structures became very similar to that with EGFP-actin. M355NA and EGFP labelling of fibres, cellular cortexes and processes were practically indistinguishable (Figure 2D). It should be stated that co-expression of EGFP-actin and M355NA-actin fusions (used for convenient comparison of the fluorescent patterns) does not lead to any difficulties in the result interpretation. Owing to the fact that transient transfection was utilized, there was always a considerable amount of cells in every transfection which expressed either EGFP-actin or M355NA-actin. Such cells serve as an internal control for the EGFP-actin fusion impact in the fluorescence pattern. In all cases, the general pattern of M355NA-actin distribution was the same in cells with and without EGFP-actin. Also, EGFP-actin alone did not produce any detectable images under conditions (filter set and exposure time) used for visualization of red signal from M355NA-actin. Control experiments with a non-related excessive free FP (EGFP or green DsRed mutant AG4 [15]) instead of asulCP-NF1 brought no improvement in M355NA-actin fluorescence pattern (results not shown).

The second helper protein tested, asulCP-NF2, worked similarly to asulCP-NF1. Addition of 10- and 50-fold excess of asulCP-NF2 resulted in a significant improvement in the labelled actin distribution (results not shown). However, asulCP-NF2 demonstrated one serious disadvantage: in its presence the brightness of red fluorescent signal from M355NA-actin was 2- to 3-fold lower than that in the case of asulCP-NF1, as deduced from analysis of pixel densities in corresponding photographs taken at the same exposure.

Tau34 labelling

Taking into account the low brightness of HOs with asulCP-NF2, we further used asulCP-NF1 helper only. We have additionally tested our method for a tubulin-binding protein Tau34 in other CHO-K1 cells. This protein was previously known to distribute properly in a cell when fused to GFP mutant S65T [16]. M355NA-Tau34 fusion protein alone in the majority cells formed aberrant bright thick fibres and did not incorporate into EGFP-Tau34-labelled fine net of tubulin microtubules (Figure 2E). Addition of helper protein asulCP-NF1 in 10-fold excess resulted in the proper binding of M355NA-Tau34 to microtubules (Figure 2F). The pattern became very similar to that of the EGFP-Tau34 distribution. Co-transfection with excessive amounts of EGFP, instead of asulCP-NF1, did not improve M355-Tau34 distribution (results not shown).

Bicistronic expression

To avoid the use of two plasmids and simplify the method, we constructed an IRES-containing vector for bicistronic expression of asulCP-NF1 and M355NA-actin in eukaryotic systems. Again, EGFP-actin fusion was used as a positive control.

Transfection of L929 fibroblasts with a plasmid expressing M355NA-actin alone (without asulCP-NF1) under IRES control resulted in an incorrect fluorescent image not corresponding to EGFP-actin distribution (Figure 2G). In contrast, cells transfected with the complete vector expressing excessive asulCP-NF1 together with M355NA-actin displayed the correct red fluorescent signal (Figure 2H). This experiment showed that a single IRES-containing vector allowed co-expression of asulCP-NF1 and M355NA-actin in the required ratio.

Characterization of HOs *in vitro*

To compare properties of M355NA tetramers with those of HOs, we investigated *in vitro* spectral characteristics of the FPs

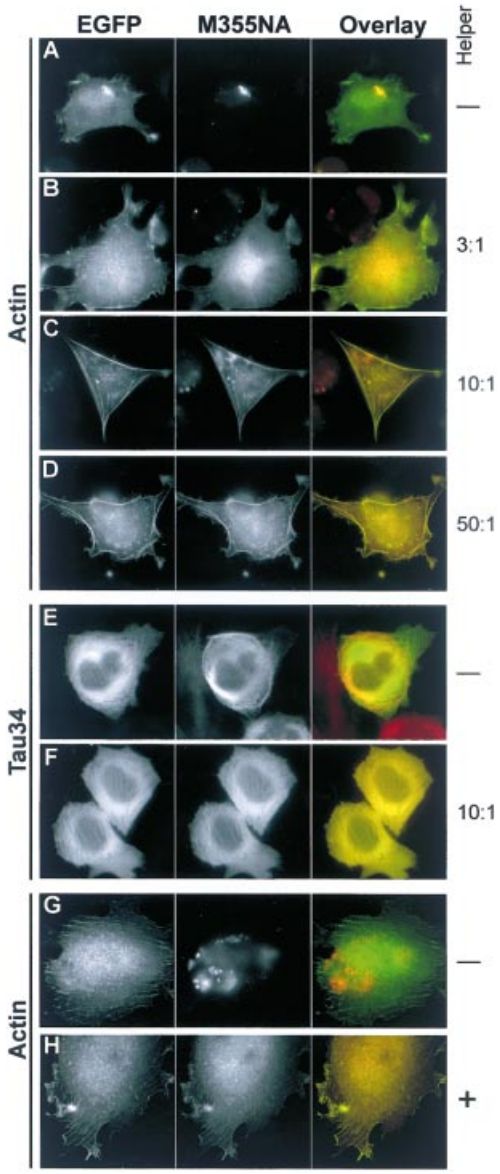

Figure 2 Expression of fusion proteins in mammalian cells

Co-transfection of eukaryotic cells with vector expressing EGFP-tagged β-actin or Tau34 was used to visualize the desired fluorescent image (positive control). The left column corresponds to the green fluorescent signal from EGFP. The middle column corresponds to the red signal from M355NA. The right column represents an overlay of the left and middle images pseudo-coloured in green and red respectively. Target proteins are designated on the left. (**A**–**D**) M355NA-actin fuse was co-expressed in L929 cells with a non-fluorescent asulCP-NF1 mutant in different proportions (**A**, no helper; **B**, **C**, **D**, 3-, 10- and 50-fold excess of asulCP-NF1). (**E**, **F**) M355NA-Tau34 labelling in CHO-K1 cells (**E**, no helper; **F**, 10-fold excess of asulCP-NF1). (**G**) IRES-dependent expression of M355NA-actin in L929 fibroblasts (negative control). (**H**) IRES-dependent expression of M355NA-actin together with Cap-dependent expression of asulCP-NF1 in L929 fibroblasts.

expressed in mammalian cells. To purify the proteins, a vector encoding M355NA with a His_6 tag at the N-terminus was constructed. Cells were transfected with His_6-M355NA alone or in conjunction with a 50-fold excess of asulCP-NF1 or asulCP-NF2. On protein maturation, His_6-M355NA, HO1 (with asulCP-NF1) and HO2 (with asulCP-NF2), containing His_6-M355NA, were purified by metal-affinity resin from corresponding cell lysates. Gel electrophoresis showed approximately

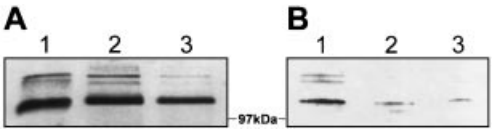

Figure 3 Pseudo-native protein gel electrophoresis of His_6-M355NA and its HOs

Protein samples were purified from CHO-K1 cells by metal-affinity chromatography, concentrated and applied without heating to native SDS/PAGE (10% gel). Lane 1, His_6-M355NA along; lane 2, HO1; lane 3, HO2. (**A**) Coomassie Blue staining. Major bands, approx. 110 kDa, correspond to tetramers, whereas minor upper bands probably can be attributed to the larger oligomers. (**B**) Western-blot analysis with antibodies against His_6 tag. Protein content is ten times lower than in (**A**).

Table 1 Spectral properties of the HOs and M355NA purified from eukaryotic CHO-K1 cells

	HO1	HO2	M355NA
Molar absorption coefficient per tetramer ($M^{-1} \cdot cm^{-1}$)	55000	235000	220000
Excitation maximum (nm)	572	572	572
Emission maximum (nm)	595	595	595
Relative fluorescence intensity	100	9.8	42.1
Relative fluorescence intensity/M355NA monomer	100	42.1	41.1
Quantum yield	0.51	0.22	0.21
Fluorescence anisotropy	0.29	0.32	0.29

equal yields for all three protein samples (Figure 3A). At the same time, Western-blot analysis of the protein bands with antibodies against the His_6 tag revealed significant differences in the staining intensities (Figure 3B). The His_6-M355NA tetramer band demonstrated approx. 4.5-fold higher staining level than both heterotetrameric bands as was measured by densitometric analysis. This value is well correlated with the expected 4-fold difference in case of one His_6-M355NA monomer per tetramer in HOs.

To compare fluorescence intensities, M355NA, HO1 and HO2 samples were adjusted by measuring their absorption at 540 nm. Fluorescence was then excited at this wavelength. Surprisingly, HO1 produced the brightest emission, whereas M355NA and HO2 were 2.4- and 10.2-fold dimmer respectively (Table 1). As measured for *E. coli*-expressed proteins, M355NA and asulCP-NF2 produce very similar absorption curves and molar absorption coefficients (55000 $M^{-1} \cdot cm^{-1}$ for M355NA and 60000 $M^{-1} \cdot cm^{-1}$ for asulCP-NF2). Thus in the case of HO2 more than 75% of absorbed light falls on the non-fluorescent helper, whereas HO1 absorbs light only due to fluorescent M355NA. Taking into account this structural difference and adjusting amounts of M355NA in HO1 and HO2, we must divide the 10.2-fold difference mentioned above by a factor 4.3. Thus the true difference in fluorescence intensities for HO1 and HO2 was calculated to be 2.4-fold, which corresponds well to the data from cell labelling.

Interestingly, fluorescence quantum yields for M355NA and HO2 were found to be practically the same but they are significantly less than that for HO1. The characteristic feature of HO1 is the presence of only one chromophore-containing monomer per oligomer. Thus one can conclude that the interaction of neighbouring chromophores within a tetramer greatly reduces the fluorescence ability of M355NA.

A similar steady-state anisotropy value of 0.30 ± 0.02 was obtained for HO1, HO2 and M355NA (Table 1). It corresponds to the relative angle between transition dipoles of the absorbing and emitting states of the chromophore, $24 \pm 3°$ [13]. In the case of M355NA, this can be explained by energy transfer between non-parallel transition dipoles of neighbouring monomers similar to DsRed tetramer [17]. Indeed, the anisotropy value of 0.3 has been reported for DsRed previously [18]. However, this simple explanation cannot be applied to the HOs since they contain only a single M355NA monomer per tetramer. Thus internal rotation of the chromophore within the protein shell or rocking of the tetramer structure is suggested for the HOs. Possibly, this also occurs in the case of the M355NA tetramer but we cannot discriminate between all these possibilities in our steady-state experiments.

DISCUSSION

Very recently, when the present experimental work was almost completed, the long-awaited creation of monomeric DsRed protein, termed mRFP, was reported [19]. However, tremendous effort was required for the monomerization of the chosen DsRed protein. Monomeric mRFP1 contained as many as 33 point mutations compared with the wild-type DsRed, and was generated by several iterative rounds of site-directed and random mutagenesis. Also, problems relating to oligomerization were resolved for far-red dimeric HcRed1 by construction of the tandem form of this protein [20].

Undoubtedly beneficial for DsRed and HcRed1, these approaches require considerable effort when applied to other tetrameric FPs that can find use in some specific tasks. For instance, a very promising FP that changes colour from green to red in response to UV or blue light illumination was discovered recently [21]. Also, we generated a photoactivated mutant of asulCP that turns from non-fluorescent into red fluorescent state under green light irradiation [22].

Our HO tagging method appears to be universal to all oligomeric FPs. A non-fluorescent helper can be easily generated by a substitution of the chromophore-forming Tyr^{66}, which is invariant for all GFP-like proteins.

The approach proposed cannot solve all problems arising due to the tetrameric nature of coral's FPs. In particular, target proteins still carry a bulky 100 kDa tag that could result in their incorrect functioning. In addition, expression of a great excess of the helper protein may negatively affect cell vitality.

Besides practical applications, HOs provide a useful model for biophysical characterization of FPs. The tetrameric nature of FPs complicates data interpretation and hides the true behaviour of monomers because of their very close interactions within tetramers [17,23]. In the present study, we demonstrated that separation of M355NA monomers from other chromophore-containing protein molecules (in HO1) resulted in a drastic increase in the fluorescence quantum yield. The most plausible explanation for this effect is effective fluorescence resonance energy transfer within a tetramer that results in partial energy dissipation. Apparently, non-fluorescent asulCP-NF2 subunits can act as an energy trap in HO2 as proposed for DsRed [17,24]. A more complex argument could be suggested for M355NA. At first glance, this FP consists of the same fluorescent monomers. However the effect of green-light-induced increase in fluorescence intensity can be observed for M355NA, as for wild-type asulCP and its other mutants [4]. Approximately 1.5–2.0-fold fluorescence enhancement can be achieved for M355NA, suggesting a significant (30–50 %) portion of the protein molecules in the non-fluorescent (or low fluorescent) state. Therefore each M355NA tetramer should contain 1–2 non-fluorescent monomers, which could play the role of fluorescence quenchers.

We believe that HOs with different transparent and coloured helpers should provide unique information on the properties of tetrameric FPs in pseudo-monomeric state in single-molecule and time-resolved studies.

Note added in proof (received 22 January 2003)

A report describing successful application of a similar method to overcome the problem of DsRed fusion protein oligomerization has been recently published [25].

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